

Neutrophils Amplify the Formation of DNA Adducts by Benzo[a]pyrene in Lung Target Cells

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Inflammatory cells and their reactive oxygen metabolites can cause mutagenic effects in lung cells. The purpose of this study was to investigate the ability of activated neutrophils to modulate DNA binding of benzo[a]pyrene (B[a]P), a known carcinogen, in lung target cells. Equivalent numbers of rat lung epithelial cells (RLE-6TN cell line) and freshly isolated human blood neutrophils (PMN) were cocultured *in vitro* for 2 hr after addition of benzo[a]pyrene (0.5 μ M) or two of its trans-diol metabolites, with or without stimulation with phorbol myristate acetate (PMA). DNA adducts of B[a]P-metabolites were determined in target cells using 32 P-postlabeling; oxidative DNA damage (7-hydro-8-oxo-2'-deoxyguanosine [8-oxodG]) was evaluated by high performance liquid chromatography with electrochemical detection. Increased DNA adducts were observed in lung cells cocultured with polymorphonuclear leukocytes (PMN). Activation of PMN with PMA, or addition of more activated PMN in relation to the number of lung cells, further increased the number of adducts, the latter in a dose-response manner. Incubation with B[a]P-4,5-diol did not result in any adduct formation, while B[a]P-7,8-diol led to a significant number of adducts. Moreover, PMA-activated PMN strongly enhanced adduct formation by B[a]P-7,8-diol, but not 8-oxodG, in lung cells. The addition of antioxidants to the cocultures significantly reduced the number of adducts. Results suggest that an inflammatory response in the lung may increase the biologically effective dose of polycyclic aromatic hydrocarbons (PAHs), and may be relevant to data interpretation and risk assessment of PAH-containing particulates. — *Environ Health Perspect* 105(Suppl 5):1089–1093 (1997)

Key words: inflammation, lung, DNA adducts, benzo[a]pyrene, cocultures, neutrophils

Introduction

An association between inflammation-related and neoplastic events has been recognized for a long time (1,2). Although a number of mediators released by inflammatory cells may play a role in this process, several studies have demonstrated that reactive oxygen species (ROS) play an important role in this interaction (3–5). First, indirect mutagenic effects of inflammatory cells were demonstrated in the

HPRT gene of rat lung epithelial (RLE) cells isolated after *in vivo* exposure to low solubility particles such as toxic silica and nontoxic carbon black (6). The increase in inflammatory response paralleled the number of *HPRT* mutations in RLE cells, which suggested that inflammatory cells, and more specifically their ROS, may be responsible for the mutagenic effects of particle exposure (7). Second, precarcinogens can

be activated by the attack of ROS, or cells producing these species (5,8). Increased generation of ROS such as superoxide anion, nitric oxide, hydrogen peroxide, and hydroxyl radical (9) is a characteristic of activated inflammatory cells such as macrophages and polymorphonuclear leukocytes (PMN). *In vitro* studies have shown that activated PMN can bioactivate polycyclic aromatic hydrocarbons (PAHs), benzene, and drugs such as acetaminophen, chlorpromazine, *p*-aminobenzoic acid, and some imidazoles (3). An *in vivo* model that induced topical skin inflammation by initial application of the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA), and a second application (16 hr later) of TPA and benzo[a]pyrene (B[a]P)-7,8-diol, was used to study the effect of inflammation on metabolism. Results showed a 50% enhancement of adducts to epidermal DNA (10). Since many environmental particles contain PAHs and induce lung inflammation (11,12), we investigated whether activated neutrophils biotransform B[a]P as a model carcinogen, and whether they are able to modulate DNA binding of one of its major metabolites. To investigate the direct action of ROS on DNA, we evaluated the formation of 7-hydro-8-oxo-2'-deoxyguanosine (8-oxodG) at the same time. Studies were done in an *in vitro* model coculturing PMN and lung epithelial cells (Figure 1).

Methods

Chemicals

B[a]P-trans-4,5-diol and B[a]P-trans-7,8-diol were purchased from the National Cancer Institute (Bethesda, MD); B[a]P, phorbol-myristate-acetate (PMA), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO). Dimethylsulfoxide (DMSO) and H₂O₂ were purchased from Merck (Darmstadt, Germany). Ham's F12, HEPES, fetal calf serum (FCS), and trypsin-EDTA were purchased from Gibco/Life Technologies (Breda, The Netherlands). Catalase and superoxide dismutase (SOD) were purchased from Boehringer (Mannheim, Germany). Sources of other chemicals, i.e., for 32 P-postlabeling and DNA isolation, are described below.

Cell Culture and Cocultures

An immortalized rat lung epithelial cell line (RLE-6TN) that possesses a near-diploid

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Abbreviations used: ATP, adenosine triphosphate; B[a]P, benzo[a]pyrene; BPDE, benzo[a]pyrene diol epoxide; dAp, deoxyadenosine-3'-monophosphate; dG, deoxyguanosine; DMSO, dimethylsulfoxide; FCS, fetal calf serum; HBSS, Hanks balanced salt solution; HPLC/ECD, high performance liquid chromatography/electrochemical detection; MPO, myeloperoxidase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NP1, nuclease P1; nt, nucleotides; 8-oxodG, 7-hydro-8-oxo-2'-deoxyguanosine; PAH, polycyclic aromatic hydrocarbon; PBS, phosphate-buffered saline; PEI, polyethylenimine; PMA, phorbol-myristate-acetate; PMN, polymorphonuclear leukocytes; PNK, polynucleotide kinase; RLE, rat lung epithelial; ROS, reactive oxygen species; SOD, superoxide dismutase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

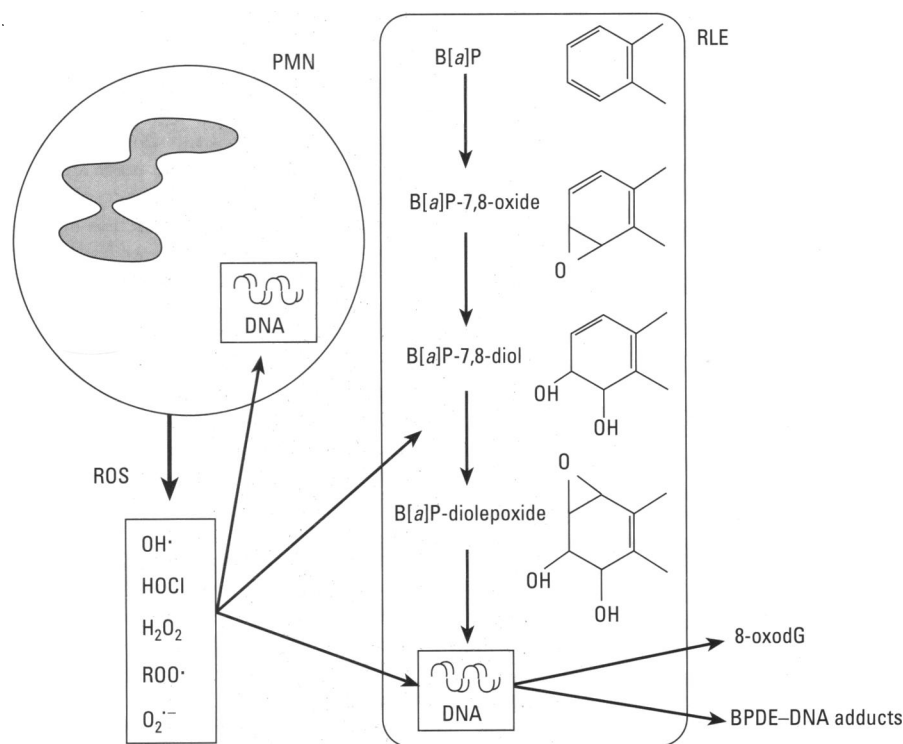


Figure 1. Illustration of the coinubation system used in this study: PMN and rat lung epithelial cells in 2-hr coinubation with B[a]P or its metabolites B[a]P-4,5-diol and B[a]P-7,8-diol. The effect parameters studied are oxidative DNA damage and B[a]P-induced DNA adducts.

genotype (13) was used to measure DNA adducts. RLE cells were grown in Ham's F12 medium supplemented with 1% 1M HEPES buffer and 5% heat-inactivated FCS; fresh medium was added to the cells every second day. At 95% confluency, culture dishes were rinsed with phosphate-buffered saline (PBS), cells were detached (trypsin-EDTA, Gibco), and seeded 1:10 in fresh culture medium. All experiments with RLE cells were performed between passages 35 and 50. Duplicate incubations were performed in separate experiments using different cell passages. Coincubations were performed at confluency of RLE cells. PMN were freshly isolated from the blood of a human male volunteer by gradient centrifugation ($800 \times g$, 20 min, 4°C) using lymphoprep (Nycomed, Oslo, Norway). To the lowest layer, 3 volumes of cold (4°C) lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3 , 10 mM EDTA, pH = 7.4, Merck) were added to lyse the erythrocytes; this procedure was repeated and PMN were suspended in Ham's F12 medium (2×10^7 cells/ml). Cell number was determined by hemacytometer chamber (Bürker) using 0.4% trypan blue to determine viability of PMN. During all steps of isolation PMN

were kept on ice to avoid activation of the cells. At the start of the incubation, PMN (2×10^7) were added to a T164 flask containing an equal amount of RLE cells (about $1.25 \times 10^5/\text{cm}^2$). All experiments except dose-response experiments (see below) used this 1:1 ratio of PMN and RLE cells. B[a]P and its diols (final concentration $0.5 \mu\text{M}$) were added in DMSO without exceeding a final concentration of 0.05% v/v. Stock solutions were kept out of the light during preparation and storage. PMN were activated with 100 ng/ml PMA. Control incubations were T164 flasks without PMN, without RLE cells, or without substrate/PMA. To determine the significance of radical-related mechanisms in DNA adduct formation in coinubations, several scavengers were evaluated. Coincubations were run as described above in the presence of catalase (5000 U/ml), SOD (2500 U/ml), or mannitol (100 mM). In these experiments, Hanks balanced salt solution (HBSS, Gibco) was used instead of Ham's F12/FCS culture medium.

Cytotoxicity

Cytotoxicity in RLE cells was determined by the MTT colometric assay developed by

Mosmann (14). The test is based on the activity of mitochondrial dehydrogenase in viable cells to convert MTT into a blue formazan dye. Cells were plated into 96-well flat-bottom culture plates (Costar, Cambridge, MA) at 1×10^4 cells/100 μl medium and cultured at 37°C and 5% CO_2 . After 2 days, fresh medium was added to each well. At confluency, samples were added and incubated at 37°C , 5% CO_2 ; subsequently, 25 μl of MTT dissolved in sterile PBS (2 mg/ml) was added and followed by a 3-hr incubation (37°C , 5% CO_2). The medium was then replaced by 200 μl DMSO, and formazan crystals were dissolved by agitation of the plate for 1 min. Absorption was measured with a microplate reader at 540 nm (Biorad, Veenendaal, The Netherlands) and cytotoxicity was calculated as described by Dong et al. (15).

DNA Isolation

After incubation for 2 hr, cells of the coinubations were gently scraped from the culture flasks with a disposable policeman (Greiner, Germany) and spun at 4°C (5 min/ $400 \times g$). Cell pellets were lysed overnight at 37°C in 5 ml NEP/SDS (75 mM NaCl, 25 mM EDTA, 50 $\mu\text{g}/\text{ml}$ proteinase K, 1% SDS), and cellular DNA was isolated as described previously (16). After precipitation of the DNA, sample concentrations were assayed spectrophotometrically. DNA was dissolved in 2 mM Tris (pH = 7.4) and stored at -20°C until analysis. Of each DNA sample, 12 μg was used for ^{32}P -postlabeling; the remaining DNA was used for high performance liquid chromatography/electrochemical detection (HPLC/ECD) analysis of oxidative DNA damage.

^{32}P -postlabeling

The ^{32}P -postlabeling assay was performed as described by Reddy and Randerath (17), with some modifications. DNA was digested using micrococcal endonuclease (0.4 U) and spleen phosphodiesterase (2.8 μg) for 3 hr at 37°C . Half of the digest was treated with 6.3 μg nuclease P1 (NP1) for 40 min at 37°C . The modified nucleotides (nt) were labeled with [$\gamma\text{-}^{32}\text{P}$]-adenosine triphosphate (ATP) (50 $\mu\text{Ci}/\text{sample}$) by incubation with 5.0 U T4-polynucleotide kinase (PNK) for 30 min at 37°C . NP1 efficiency and ATP excess were checked with an aliquot of the NP1-treated fraction by one-dimensional chromatography on poly(ethylenimine) (PEI)-cellulose sheets (solvent: 0.12 M NaH_2PO_4 , pH = 6.8 on Merck sheets). Radiolabeled

adduct nucleotide biphosphates were separated by chromatography on PEI-cellulose sheets (Machery Nagel, Düren, Germany). The following solvent systems were used: D1, 1 M NaH_2PO_4 (pH=6.5); D2, 8.5 M urea, 5.3 M lithium formate (pH=3.5); D3, 1.2 M lithium chloride, 0.5 M Tris, 8.5 urea (pH=8.0); D4, 1.7 M NaH_2PO_4 (pH=6.0). In each experiment, three standards of [^3H]B[a]P diol epoxide (BPDE)-modified DNA with known modification levels ($1:10^7$, $1:10^8$, $1:10^9$ nt) were run simultaneously. Quantitation was performed by phosphor imaging technology (Molecular Dynamics, Sunnyvale, CA). The detection limit of the assay was approximately one adduct per 10^9 nt. The remaining half of the digest was used to determine the final amount of DNA in the assay; the normal nucleotides were labeled with [γ - ^{32}P]-ATP (15 $\mu\text{Ci}/\text{sample}$) by incubation with 2.5 U PNK for 30 min at 37°C . Nucleotides were separated by one-dimensional chromatography on PEI-cellulose sheets (solvent: 0.12 M NaH_2PO_4 , pH=6.8 on Merck sheets); samples with apparent protein or RNA contamination were discarded. A deoxyadenosine-3'-monophosphate (dAp) standard (27.5 pmol/ μl) was labeled in each experiment for quantitation.

HPLC/ECD Analysis of 8-oxodG

Oxidative damage in DNA was evaluated as described previously (17), according to Floyd et al. (18), and was expressed as the ratio of 8-oxodG to deoxyguanosine (dG). The lower limit of detection was 40 fmol absolute for 8-oxodG, or 1.5 residue/ 10^6 dG, which required a minimum yield of 35 μg DNA per sample.

Results

Rat Lung Epithelial Cells

Cytotoxicity experiments indicated that B[a]P and B[a]P-7,8-diol were not cytotoxic in a concentration range from 0.05 to 5 μM , and further experiments used a maximum concentration of 5 μM (cytotoxicity < 10% for both chemicals). A 2-hr exposure to H_2O_2 up to concentrations of 100 μM was not cytotoxic to RLE cells (< 3% at 100 μM). However, in RLE cells incubated with 500 μM H_2O_2 , cytotoxicity was 50%.

^{32}P -postlabeling profiles of DNA from RLE cells incubated with B[a]P and B[a]P-7,8-diol showed a predominant adduct spot (90% of total adducts) that cochromatographed with BPDE-DNA standard (Figure 2). This putative BPDE-DNA

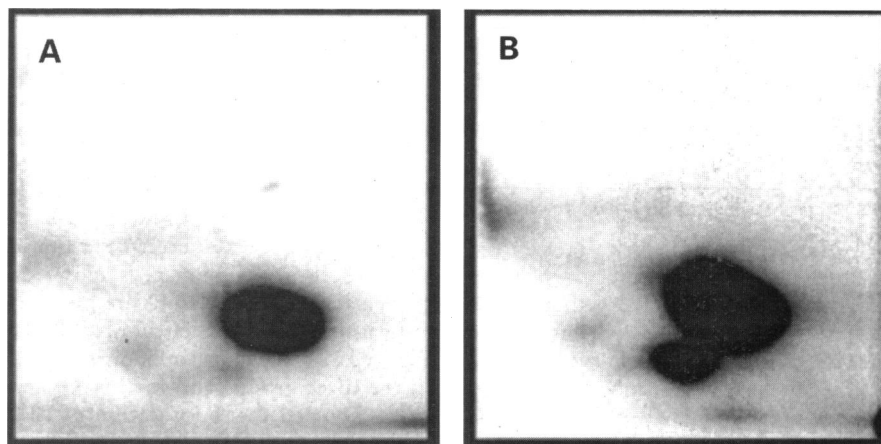


Figure 2. Two-dimensional chromatograph illustrating the adduct profile in coincubations of RLE cells and PMN in the (A) absence or (B) presence of PMA. The origin is located at the lower right corner and was excised before analysis.

Table 1. BPDE-DNA levels in RLE cells incubated for 2 hr with B[a]P, B[a]P-4,5-diol, and B[a]P-7,8-diol.

Substrate	0.05 μM	0.5 μM	5 μM
B[a]P	9.9 ± 2.8^a	7.6 ± 0.8	5.2 ± 0.6
B[a]P-7,8-diol	33.3 ± 9.5	460.4 ± 240	1898.3 ± 220.8
B[a]P-4,5-diol	ND	ND	ND

ND, not detectable. ^aNumber of DNA adducts/ 10^8 nucleotides.

adduct has been quantitated and will be referred to as the BPDE-DNA adduct. BPDE-DNA adduct formation is shown in Table 1. No dose-dependent increase in adducts was observed in RLE cells incubated with B[a]P for 2 hr. Incubation with B[a]P-7,8-diol for 2 hr resulted in higher adduct levels compared to equimolar concentrations of B[a]P, and the increase in BPDE-DNA adduct levels was also dose dependent (Spearman $r = 0.96$, $p < 0.05$). No adducts were detected by ^{32}P -postlabeling in RLE cells incubated with B[a]P-4,5-diol. PMA had no effect on BPDE-DNA adduct formation in RLE cells exposed to 0.5 μM B[a]P, i.e., 2.6 ± 1.6 adducts/ 10^8 nt (without PMA) versus 2.7 ± 1.7 adducts/ 10^8 nt (with PMA).

Background oxidative DNA damage, as measured by 8-oxodG/dG, was readily detectable in RLE cells. A 2-hr exposure to 10 μM H_2O_2 resulted in a slight but not significant increase of the 8-oxodG/dG ratio (142% compared to the control incubations).

Coincubations

Figure 3 shows the calculated BPDE-DNA adduct levels in the coincubations exposed to B[a]P at different ratios of PMN to RLE

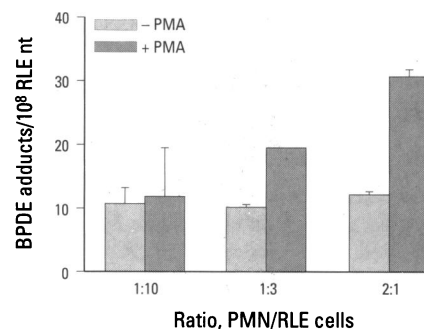


Figure 3. The calculated amount of BPDE-DNA adducts in RLE cells coincubated for 2 hr with PMN at different ratios and 0.5 μM B[a]P.

cells. RLE cells and PMN were not separated before DNA was isolated. The adduct levels shown in Figure 3 were corrected for the different number of PMN; previous experiments demonstrated that in PMN exposed to B[a]P, adduct levels are negligible. Figure 3 shows that using unstimulated PMN, no enhanced adduct formation was observed in RLE cells with an increasing number of PMN. However, using activated PMN, adduct formation increased with an increasing number of PMN. As illustrated in Figure 3, PMN activation resulted in a marked enhancement of adducts. An even stronger enhancement of adducts in coincubations was observed by PMN activation of B[a]P-7,8-diol (Table 2). Typical chromatographs of these experiments are shown in Figure 2. No BPDE-DNA adducts were detected in PMN or in coincubations exposed to B[a]P-4,5-diol.

Activation of the neutrophils' oxidative burst with PMA also resulted in significantly higher 8-oxodG/dG ratios within their own

Table 2. BPDE–DNA levels in PMN or coin incubations of PMN + RLE cells (1:1) incubated for 2 hr with 0.5 μ M B[a]P-7,8-diol and PMA.

	PMN	PMN + RLE
– PMA	50.3 \pm 0.14	177.7 \pm 111.6
+ PMA	360.3 ^a	818.8 \pm 19.9

Data represent mean and SD of two experiments, except ^a*n* = 1.

DNA (i.e., 188.5%, *n* = 3 experiments). In unstimulated PMN, B[a]P was able to enhance oxidative DNA damage (118.5%, *n* = 3). However, in coin incubations, no significant effects of PMA or B[a]P or its diols on 8-oxodG/dG were observed (data not shown).

In Figure 4, the effect of various radical scavengers on BPDE–DNA adduct formation in coin incubations exposed for 2 hr to B[a]P-7,8-diol is shown. In comparison to the control incubation in HBSS, incubation in culture medium supplemented with FCS reduced BPDE–DNA adducts similar to catalase-supplemented HBSS. In the presence of both catalase and SOD, BPDE–DNA adduct levels further decreased, while mannitol or SOD alone had no scavenging effect. The MTT assay showed no cytotoxicity of SOD and/or catalase at the concentrations used (< 5%). However, 100 mM mannitol reduced the viability of RLE cells by 27%.

Discussion

In this study we showed that activated neutrophils are able to modulate biotransformation of B[a]P and DNA binding of its crucial genotoxic metabolite BPDE in an *in vitro* coin incubation model of neutrophils and lung epithelial cells. In coin incubations exposed to B[a]P and stimulated with PMA, BPDE–DNA adduct levels

appeared to be correlated with the number of PMN (Figure 2). These results suggest that during *in vivo* exposure to B[a]P, the formation of BPDE–DNA adducts in the lung might be related to the extent of inflammation i.e., the percentage of PMN. Since ultrafine particles induce rapid PMN influx after inhalation, these data might be of significance to the interpretation of health effects in environmental exposure to particulates (7). Our results are probably not caused by a direct effect of PMA on RLE cells, as PMA concentration was equal in the three groups and further experiments showed that stimulation of RLE cells (without PMN) with PMA did not increase the level of BPDE–DNA adducts. Our data also indicate that PMN are unable to metabolize B[a]P to B[a]P-7,8-diol, probably as a consequence of their negligible CYP450 activity. No adducts were detected by ³²P-postlabeling on incubation with B[a]P-4,5-diol in the coin incubations, or in either RLE cells or PMN alone, which agrees with the observation that this diol cannot form DNA adducts (19). Exposure to B[a]P-7,8-diol, however, resulted in high adduct levels in PMN, and stimulation of PMN with PMA further increased these levels both in PMN and in coin incubations. These observations might be explained by the fact that B[a]P-7,8-diol can be metabolized to BPDE by several metabolic routes other than CYP450, including myeloperoxidase (MPO), lipid-peroxidation products (20,21), and probably also by ROS. During activation of PMN (e.g., by PMA), excessive formation of these products occurs. However, our results do not elucidate whether the metabolic conversion of B[a]P-7,8-diol occurs extracellularly by ROS or MPO or intracellularly by lipid peroxidation. In coin incubations adduct levels were even higher, both in the absence and presence of PMA, in comparison to PMN (Table 2). This is most likely the consequence of a higher metabolic capacity (including CYP450 activity) in RLE cells. The increase in adduct levels by PMA in coin incubations may also result from the extracellular actions of MPO and/or ROS released by PMN. Furthermore, ROS released by activated PMN may cause lipid peroxidation of RLE membranes, which leads to intracellular peroxyradicals, which are known to activate B[a]P-7,8-diol *in vitro* as well as *in vivo* (22). Addition of scavengers demonstrated the role of ROS in DNA adduct formation of BPDE, because catalase, as well as catalase plus SOD, but not SOD

alone, were able to reduce BPDE–DNA adduct formation. Comparison of incubations in HBSS versus culture medium showed that the effects observed in coin incubation models occur even in the presence of the scavenging effects of fetal serum. Thus, the effects observed in coin incubations would probably be much stronger if HBSS was used, but they indicate that the effects we observed *in vitro* are also more likely to occur *in vivo* despite scavenging action of antioxidants. Our results are in agreement with data of Kensler and co-workers, who reported increased DNA adduct formation in mouse keratinocytes exposed to [³H]-B[a]P-7,8-diol in the presence of TPA-activated PMN (10). This DNA binding may be MPO dependent, as supported by the ability of azide to reduce this binding (5,8).

Parallel analysis of oxidative DNA damage showed that stimulation by phorbol ester caused enhanced ratios of 8-oxodG/dG in PMN, which is in agreement with previous observations (18,23) but in contrast to a recent study (24). In all experiments 8-oxodG/dG ratios were higher in PMN than in coin incubations, which indicates that PMN are more susceptible to oxidative damage than RLE cells. This can be explained by the high resistance of RLE cells to H₂O₂, and the high local concentrations of ROS in or around PMN. Exposure to B[a]P in the absence of PMA resulted in increased 8-oxodG/dG ratios in PMN but not coin incubations. This oxidative effect of B[a]P has been reported by Mauthe et al. (25), and might be explained by one-electron oxidation of B[a]P (21). However, this process is negligible compared to the generation of ROS by activated PMN.

In conclusion, this coin incubation model is a suitable *in vitro* system to study mechanisms of PMN-mediated DNA damage in lung target cells. The results of our experiments clearly show that the genotoxic action of B[a]P toward lung epithelial cells is not caused solely by classic biotransformation processes, but also involves ROS produced by PMN. Moreover, if applicable to particle-associated PAHs our data might promote testing and evaluation of many environmental airborne particulates with regard to genotoxic outcomes in the lung. Experiments are currently in progress to elucidate the responsible ROS involved in this model. However, experiments are needed to investigate whether the dose–response observations regarding number of PMN can be confirmed in an *in vivo* model of lung inflammation and related genotoxicity.

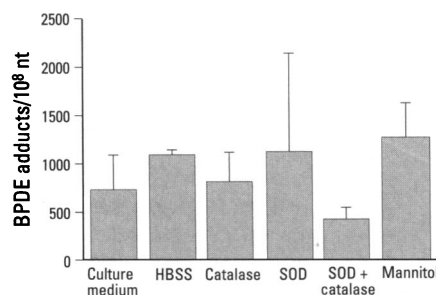


Figure 4. Effect of culture medium (including 5% FCS) and antioxidants catalase (5000 U/ml), SOD (2500 U/ml), and mannitol (100 mM) on BPDE–DNA adduct formation in RLE cells coin incubated with PMA (100 ng/ml)-activated PMN and B[a]P-7,8-diol (0.5 μ M).

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